Cytological and Enzymological Characterization of Adult Human Adipocyte Precursors in Culture

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ABSTRACT Cell strains were derived from the stromal-vascular fraction of human omental adipose tissue and grown in culture. Since the purpose of this study was to isolate adipocyte precursors from adults, the cells were obtained from nonobese patients 40–60 yr of age. After treatment of adipose tissue with collagenase, mature adipocytes were separated from stromal-vascular fraction cells, and cell strains of the latter replicated in culture with a doubling time of 40–60 h. They were initially fusiform; upon reaching monolayer confluency, they accumulated lipid and became rounder. Skin fibroblasts from the same patients and grown under the same culture conditions remained fusiform and did not accumulate lipid. The stromal-vascular fraction cells of adipose tissue may be fibroblasts with the potential to become adipocyte precursors. Subcellular preparations of the cells grown from the stromal-vascular fraction revealed lipoprotein lipase activity (characterized by such properties as inhibition by 1 M NaCl) that was not detectable in skin fibroblasts. The overall specific activity of the enzymes that catalyze triglyceride synthesis was 15 times higher and that of fatty acid synthetase was 2 times higher in the cells cultured from the stromal-vascular fraction. The difference was significant in each case. Conversely, when isolated mature adipocytes were cultured, they lost considerable lipid and acquired morphological characteristics similar to those of stromal-vascular fraction cells. Thus, adipose tissue stromal-vascular fraction cells acquire in culture many of the morphological and enzymological characteristics of mature fat cells.

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INTRODUCTION

Knowledge regarding the cytological and biochemical aspects of fat cell differentiation is limited. In particular, information is lacking regarding the enzymes that are involved in the specialized functions subserved by adipocytes.

The differentiation of precursor cells into mature adipocytes has been studied in prepubertal rats by injecting radioactive thymidine in vivo and by incubating radioactive thymidine with adipose tissue in organ culture. Maturation of cells derived from the stromal-vascular fraction (SVF) [1] of adipose tissue occurred, as indicated by a rise in DNA specific radioactivity in the adipocyte fraction (1–4).

It has been proposed that fat cells originate in vivo from fibroblasts (5), one specific clones of 3T3 fibroblasts can develop "preadipose" characteristics in culture (6). The SVF cells of adipose tissue, which have the potential to differentiate into fat cells and which will be described in this paper, may originate as specialized fibroblasts.

A number of investigators have deduced that new fat cells cannot be formed in white adipose tissue of mammals, including man, after puberty (7–11). These deductions were based on methods of cell preparation and counting that would have excluded immature, dedifferentiated, and mature adipocytes devoid of appreciable fat (12, 13).

Recently, cells derived from adipose tissue of human adults have been grown in culture and shown to have certain characteristics different from those of skin fibroblasts (14, 15). Poznanski and co-workers (16) have succeeded in establishing putative adipocyte precursor strains in culture. These cells, which were derived from the SVF of adult human adipose tissue, initially re-

[1] *Abbreviation used in this paper:* SVF, stromal-vascular fraction.

sembled skin fibroblasts but in later culture accumulated more triglyceride and incorporated more radioactive glucose into lipids. Despite appreciable lipid accretion, the SVF cells did not acquire all the morphological characteristics of mature adipocytes. Because of the possibility that they were adipocyte precursors, we have characterized these cells with particular reference to the activity of enzymes subserving specialized functions characteristic of mature fat cells. The evidence which will be presented indicates that these cell strains are indeed adipocyte precursors that have the capability to replicate and differentiate in culture.

METHODS

Source and preparation of cells. Tissue from patients 40–60 yr of age was obtained at the time of abdominal surgery; the patients were not obese according to Metropolitan Life Insurance tables. They were fasted for 9–12 h and then received a slow infusion of 5 g/dl dextrose for 1–2 h preoperatively and up to the time of fat and skin excision.

Omental adipose tissue and abdominal skin samples were resected at the beginning of each operation and immersed in Hanks' balanced salt solution at 0–4°C. Within 1 h, the adipose and skin tissues were minced and treated with collagenase (1 mg/ml, Worthington Biochemical Corp., Freehold, N. J.) at 37°C for 15 and 60 min, respectively (12). After centrifugation, the digested stromal-vascular fraction of adipose tissue and the skin preparations were suspended in a chemically defined (alpha) medium (17) supplemented with 20% fetal calf serum (Flow Laboratories Inc., Phoenix, Ariz.) and with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Cell culture of stromal-vascular fraction cell strains and skin fibroblasts. On the average, 2×10^5 SVF cells of adipose tissue or skin fibroblasts were plated into either Leighton Tubes (Bellco Glass, Inc., Vineland, N. J.) for cytological studies or Falcon 25-cm² tissue culture flasks for biochemical studies (Falcon Plastics, Division of Bioquest, Oxnard, Calif.). Cells were cultured to monolayer confluency in a humidified incubator (Masterline, Forma Scientific, Marietta, Ohio) at 37°C, pH 7.4, in the presence of 5% CO_2. The cells were washed, and the growth medium was changed every 2nd day. The cells were detached by incubation with trypsin (0.25 g/dl, pH 7.4, Flow Laboratories) at 37°C for 30 min and centrifuged at 700 *g*, 15 min, 22°C; the resulting pellets were washed thoroughly and used for subculture. The first three subcultures were used for morphological, chemical, and enzymological studies.

Cell culture of mature adipocytes. To obtain isolated mature fat cells free of stromal-vascular elements, the floating layer prepared after collagenase digestion was filtered through a 200–250 μm nylon sieve. The filtered cells were washed five times with Hanks' balanced salt solution and filtered each time. The cells in the last filtrate were examined microscopically, and only mature adipocytes isolated free of any detectable contamination with stromal-vascular elements were allowed to adhere between two glass coverslips at 22°C for 15 min. The specimens were then introduced into Leighton tubes that contained 1 ml of the previously described growth medium. When the cells lost their spherical shape, they were subcultured. The first three subcultures were studied.

Cell staining. Cells were stained with hematoxylin, and cellular lipids were stained with Oil Red O.

Growth curves. Cells were prepared as described for subculture and resuspended in 1 ml of Isoton (Coulter Electronics, Inc., Hialeah, Fla.), and diluted portions were counted using a Coulter Counter model F.

Protein determination. Adipose tissue SVF or skin fibroblasts were dissolved with 0.5 M KOH, and protein was determined by a microbiuret procedure (18).

Lipoprotein lipase assay. Cells were disrupted by sonication (five intervals of 30 s at 50 kcycle and 0–4°C, Biosonik III Bronwill Scientific, Rochester, N. Y.) in 0.1 M NH_4OH-NH_4Cl, pH 8.6, containing 0.2 U/ml of sodium heparin. After centrifugation at 800 *g* at 4°C for 20 min, the supernates were used as the enzyme source. The substrate mixture consisted of 0.1 M NH_4OH-NH_4Cl, pH 8.6, 7.5 μM glyceryl [^{14}C]trioleate (Amersham-Searle Corp., Arlington Heights, Ill., 28 Ci/mol), 24 mg of bovine serum albumin [Armour Fraction V, Armour Pharmaceutical Co., Chicago, Ill., free of fatty acids (19)], and 0.13 ml of a 1% (vol/vol) aqueous Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.) solution in a final volume of 0.6 ml. Sonication was performed as described for cell disruption. The sonicate was incubated with 0.1 ml of human serum at 37°C for 10 min. The enzyme preparation was incubated with either NaCl (final concentration 1 M) or buffer at 4°C for 30 min. The reaction was started by adding enzyme preparation which brought the final volume of the assay mixture to 1.0 ml. Incubations were conducted at 37°C for 30 min. Control tubes were devoid of enzyme preparation. The released [^{14}C]oleate was extracted with the mixture of Dole and separated from triglyceride and lower glycerides by converting the free acid to potassium [^{14}C]oleate with KOH. [^{14}C]Oleic acid was recovered from the lower phase by acidification and extraction with petroleum ether. After evaporating the organic solvent under N_2, the fatty acid was dissolved in heptane, and radioactivity was counted in a Nuclear Chicago Mark II liquid scintillation spectrometer model 6847 (Nuclear-Chicago Corp., Des Plaines, Ill.) by using Bray's solution (20). In these and the other enzyme assays to be described, only initial rates were determined.

Fatty acid synthetase assays. The fatty acid synthetase activity present in the 800 *g* supernates was determined by two different methods. One involved the incorporation of [2-^{14}C]malonyl-CoA into long-chain fatty acids in the presence of optimal concentrations of malonyl-CoA, acetyl-CoA, glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, and dithiothreitol in K phosphate buffer (pH 7.0, 37°C); control assays were devoid of the NADPH-generating system (21). The second method involved the spectrophotometric (Gilford recording spectrophotometer 2400-S, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) quantification of NADPH oxidation in the presence of optimal concentrations of malonyl-CoA, acetyl-CoA, NADPH, and dithiothreitol in potassium phosphate buffer (pH 7.0, 27±1°C); control assays were devoid of malonyl-CoA (21).

Esterification of sn-glycero-3-phosphate. The cell-free system used to study the enzymes which catalyze triglyceride synthesis contained optimal concentrations of substrates and cofactors in the presence of the enzyme preparation (800 *g* supernate); the constituents were 0.1 M potassium phosphate (pH 7.0), 3 mM [U-^{14}C]sn-glycero-3-phosphate (1 μCi per assay), 0.08 mM K palmitate, 3.3 mM ATP, 2 mM $MgCl_2$, 0.08 mM CoA, 0.05 M KCl, 0.01 M 2-mercaptoethanol, and enzyme preparation to a total volume of 0.5 ml. Control assays were devoid of ATP. Incubations were conducted at 37°C for 30 min.

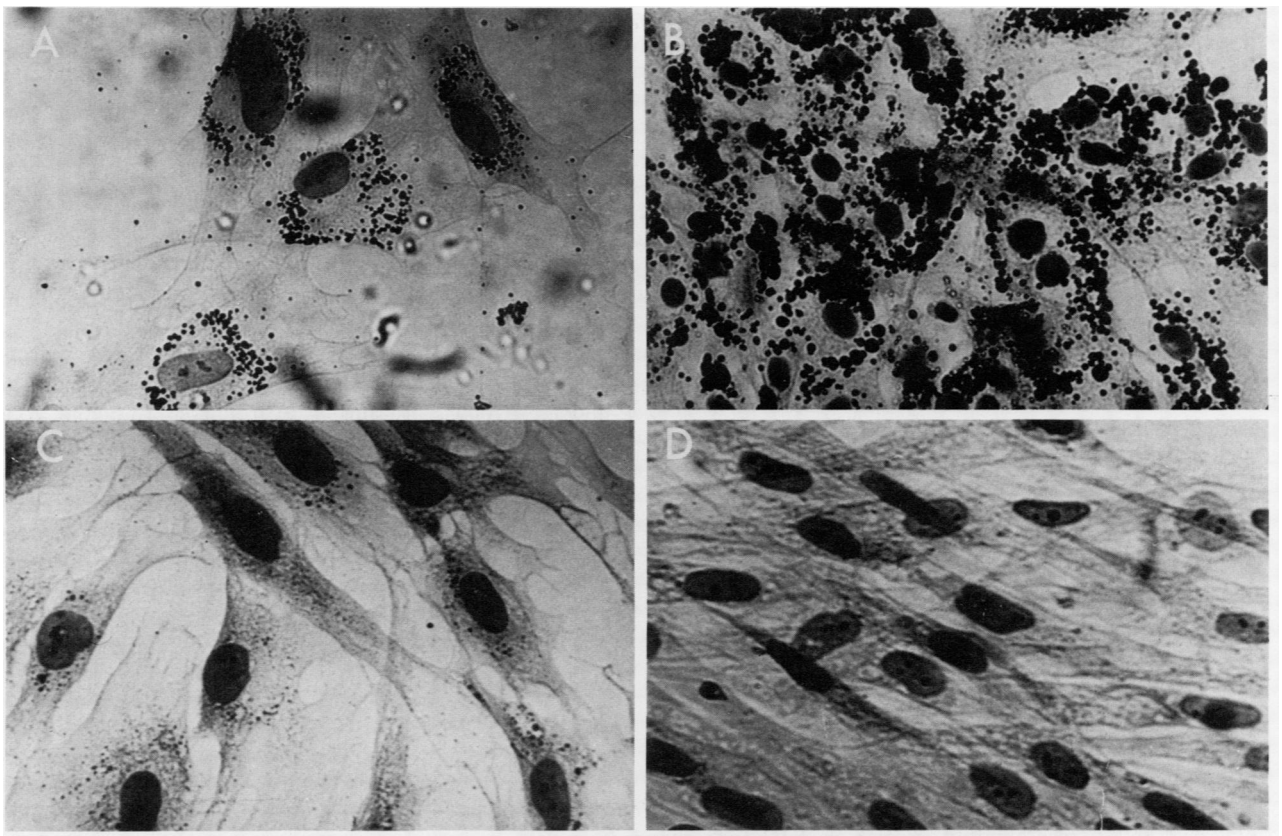

FIGURE 1 Morphology of human omental adipose tissue stromal-vascular fraction cells compared with skin fibroblasts from the same patient and of the same culture age. Magnification × 400. Stromal-vascular fraction cells on days 6(A) and 12(B). Skin fibroblasts on days 6(C) and 12(D). Lipids (black cytoplasmic particles) were stained with Oil Red O, the nucleus and cytoplasm with hematoxylin.

The newly formed radioactive glycerolipids were extracted by the method of Bligh and Dyer (22) by using methanol that contained 0.01 M rac-glycero-phosphate and by employing 0.1 M HCl instead of H_2O. Triglycerides were separated from the other glycerolipids by thin-layer chromatography on Silica Gel H (Silica Products Co., Inc., Guion, Ark.) as previously reported (23). 50 μg each of authentic 1, 2-diacyl-sn-glycero-3-phosphate, diglyceride, and triglyceride were used as "carriers". After visualization with I_2, the Silica containing the lipid fractions was scraped, and the radioactivity was counted.

RESULTS

Morphological studies. The morphological characteristics of cell strains derived from the stromal-vascular fraction of adult human adipose tissue are illustrated in Fig. 1. They are compared to those of skin fibroblasts obtained from the same patient and of the same culture age. These cells were in first subculture; identical results with regard to these or any other properties were obtained with any of the first three subcultures. These revealed homogeneous cell strains. So far, we have carried these through 20 passages without any evidence of alterations in replicative rate or morphology.

The characteristic cell isolated from the SVF of adipose tissue by our methods initially had sparse cytoplasmic lipid inclusions, a central nucleus, and a fusiform shape (Fig. 1A). Upon reaching monolayer confluency, the cells acquired many lipid inclusions and became rounder (Fig. 1B). In contrast, the skin fibroblasts multiplied and reached monolayer confluency without any appreciable lipid accumulation or morphological change (Fig. 1C and D).

Fig. 2A illustrates that adipose tissue SVF cells and skin fibroblasts had similar replicative rates; both cell strains multiplied with a doubling time of 40–60 h. There was also no significant difference in their protein concentrations (Fig. 2B) during a growth period encompassing one generation.

Enzymological studies. Subcellular preparations from stromal-vascular fraction cells revealed reproducible lipoprotein lipase activity (Fig. 3). As in the case of the lipase from mature adipocytes, the enzyme was inhibited completely by incubation with 1 M NaCl. Skin fibroblasts did not have any detectable activity (Fig. 3).

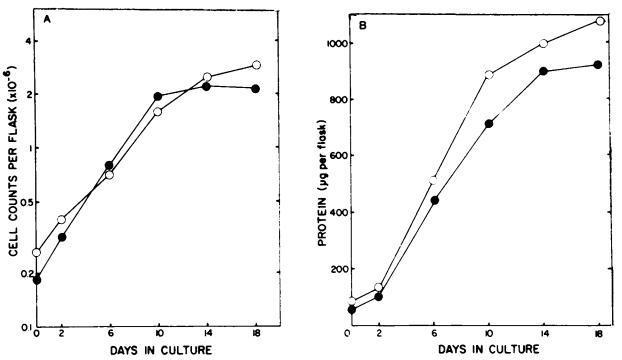

FIGURE 2 Cell counts (A) and protein concentrations (B) during replication of stromal-vascular fraction cells (●—●) and skin fibroblasts (○—○). The cell counts and protein concentrations encompassed one growth period in culture and are representative of 20 different cell strains.

Small differences in such details of cell culture as the precise age at the time of harvest probably accounted for variations in enzymatic activity; these led to an increase in the standard error of the mean. Control skin fibroblasts that were treated under identical culture conditions were grown for each study, and the difference in lipoprotein lipase activity between the two types of cells was consistent.

As shown in Fig. 4, the mean specific activity of fatty acid synthetase was twofold greater in SVF cell than in skin fibroblast preparations. Analysis of paired samples by the t test indicated a significant difference between SVF cells and fibroblasts at $P < 0.01$.

The mean overall specific activity of the enzymes that catalyze triglyceride synthesis from sn-glycero-3-phosphate and fatty acids was 15-fold higher in SVF cell than in skin fibroblast preparations (Fig. 4). The same statistical considerations and analysis as described for fatty acid synthetase applied to these enzymes. The difference between the two cell strains was significant at $P < 0.025$.

Mature adipocytes were isolated free of any detectable stromal-vascular elements (Fig. 5A). As described under Methods, precautions were taken to avoid contamination with stromal-vascular elements; nonetheless, one cannot exclude absolutely a small degree of contamination. Upon culture, they lost considerable lipid, the nuclei became more central, and the cells became elongated in shape (Fig. 5B). At this stage, they resembled the more differentiated SVF cells observed in culture (Fg. 1B). While mature fat cells did not replicate initially, after assumption of a more elongated contour, they multiplied with the same doubling time as that of SVF cells.

DISCUSSION

This study has demonstrated the presence in adult human adipose tissue of cells that have the potential to

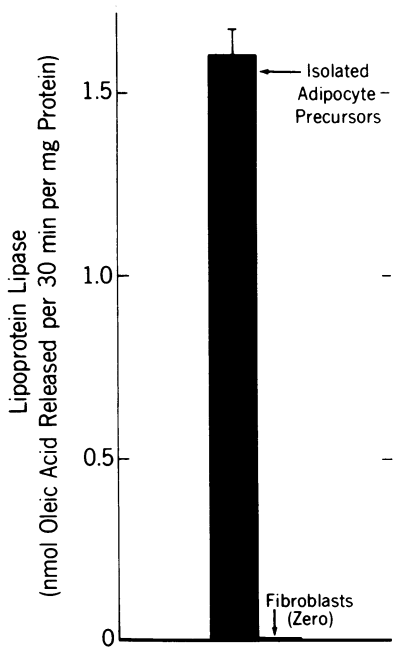

FIGURE 3 Determination of lipoprotein lipase activity. The mean and its SE are shown in the case of stromal-vascular fraction cells (isolated adipocyte precursors). Activity was undetectable in skin fibroblasts.

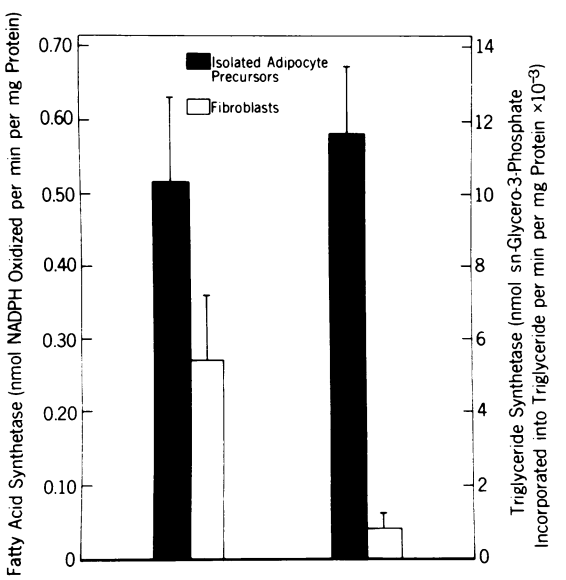

FIGURE 4 Determination of enzyme activities in stromal-vascular fraction cells (isolated adipocyte precursors) and skin fibroblasts. The difference between the two cell strains by the paired t test was significant for each enzyme system, for fatty acid synthetase at $P < 0.01$ and for triglyceride synthetase at $P < 0.025$. In each case, the mean and its SE are shown.

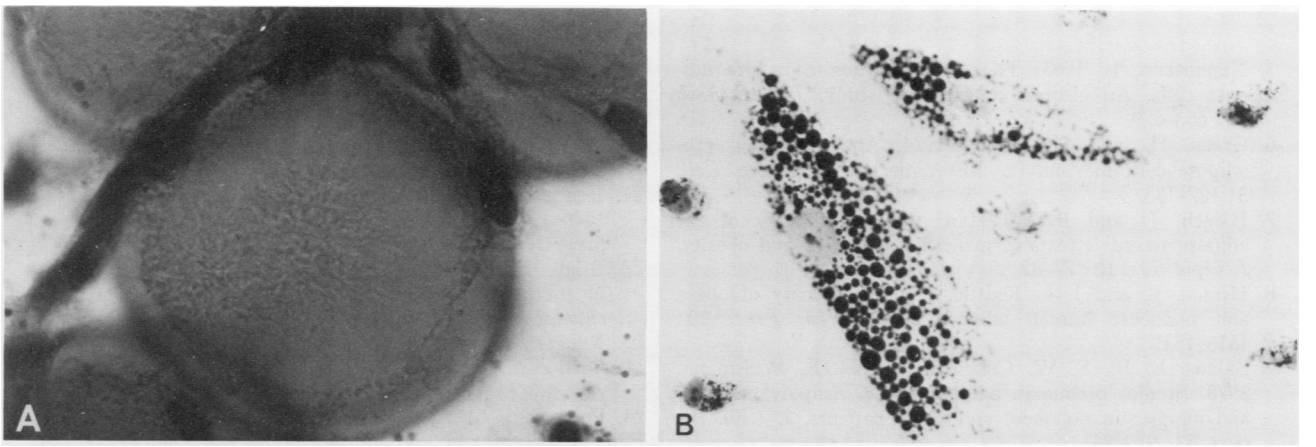

FIGURE 5 Morphology of isolated adipocytes on days 2(A) and 12(B). Magnification × 400. Lipids (black cytoplasmic particles) were stained with Oil Red O, the nucleus and cytoplasm with hematoxylin.

multiply and acquire some morphological and biochemical characteristics similar to those of mature adipocytes.

The activity of enzymes involved in adipocyte lipid assimilation and synthesis was determined in the putative fat cell precursors (stromal-vascular fraction cells) and compared to that in skin fibroblasts from the same patient and of the same culture age. The specific activity of all the enzymes studied was either significantly higher in the cells derived from the stromal-vascular fraction of adipose tissue, or, in contrast to these cells, activity was undetectable in skin fibroblasts. Our work corroborates previous morphological findings (16). Furthermore, the enzymological studies provide strong additional evidence for the relatedness of the SVF cells to mature adipocytes.

Mature fat cells have been shown to undergo marked morphological alterations in culture. They acquired the cytological characteristics of differentiating SVF cells, observations that provide additional evidence for their cognate nature. Similar changes have been observed in the case of human subcutaneous fat cells (24). The possibility that these cells were derived from stromal-vascular elements that contaminated the mature adipocyte preparations cannot be absolutely excluded. The reversion of mature adipocytes to a primordial form may involve a process of dedifferentiation. The current studies have also revealed that, having acquired their new characteristics, these cells replicated in culture at the same rate as the SVF cells. It is possible that the immature adipocytes present in the stromal-vascular fraction of adult human adipose tissue are either adipocyte precursors that were never mature or fat cells that have acquired primordial properties, or that both cell types exist.

The presence of immature fat cells in adult human adipose tissue suggests the potential for adipocyte differentiation in adults. Unpublished work in which rat adipocyte precursors were labeled in their DNA and differentiated into mature fat cells after re-implantation into the same adult donor animals, supports this proposal.[2] Further support is gained by a study which indicates that the epididymal adipose tissue of adult guinea pigs enlarges as a result of fat cell multiplication (25). A few investigations have revealed, moreover, that adult animals acquire an increased number of fat cells during certain nutritional states and when exposed to cold temperatures (26–31). While extrapolation of our findings in cell culture to in vivo events may not be warranted, one might speculate that the adipocytes of human adults are not static; under conditions of steady body fat content, a slow, continuous replacement of mature adipocytes may occur. The fat cell precursors characterized in this study should provide important information about the factors that regulate the cellularity and size of adipose tissue.

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